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(71)(72) Applicants and Inventors: BAYLINK, David, J. [US/US]; 1534 Fern Avenue West, Redlands, CA 92373 (US). LINKHART, Susan [US/US]; 220 Gabrielle Way, Redlands, CA 92374 (US).

(74) Agents: PARMELEE, Steven, W. et al.; Townsend and Townsend Khourie and Crew, One Market Plaza, 20th Fl., Steuart Tower, San Francisco, CA 94105 (US).

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(57) Abstract

Peptides which contain epitopes that mimic epitopes of the amino terminal propeptide of α1 Type I collagen, and antibodies which bind the epitopes, are useful in monitoring bone formation. Assays which employ the peptides and antibodies thereto are particularly useful in diagnosing and monitoring bone related disorders, such as osteoporosis and Paget's disease, among others.

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AMINO PROCOLLAGEN 1(I) PEPTIDES

Field of the Invention

This invention relates to methods for determining bone formation rates by measuring levels of the amino terminal propeptide of procollagen alpha (α)1 chain of Type I collagen.

Background of the Invention

Bone formation in vertebrates is a dynamic process involving continuous production of bone and continuous bone resorption. Osteopenia is a general term used to describe any bone-wasting disease in which the rate of bone resorption is greater than the rate of bone formation. Osteoporosis results from a progressive net loss of skeletal bone mass due to an increase in bone resorption exceeding bone formation. Osteoporosis afflicts nearly 20 million people in the United States alone, and total costs from osteoporosis-related injuries amount to at least $7 billion annually (Barnes, Science 236:914 (1987)). A major difficulty in monitoring the disease is the lack of a specific assay to measure acute changes which may occur from various treatment regimens. There is a need to have an easy, reliable test for bone formation.

Type I Collagen is unique to connective tissues and is a major component in bone, among other tissues. The normal synthesis and breakdown of this collagen type can be altered during the pathogenesis of many kinds of disease, including osteoporosis. Because bone is a metabolically active tissue throughout life, indicators of Type I collagen turnover could be useful as a marker in metabolic bone disease. However, the major means for estimating the metabolic rate of bone collagen

has been to quantify the urinary excretion of hydroxyproline, which is derived from collagenous proteins. This test has proven tedious, associated with several sources of error, and not specific for Type I collagen. Azria, Calcif. Tissue Int. 45:7-11 (1989).

Each Type I collagen fiber is composed of three long, helical polypeptide chains (α chains) that bind tightly to each other. Each Type I collagen polypeptide is synthesized as a larger procollagen molecule, containing additional sequences at both the amino and carboxy terminals. It appears that the large amino and carboxy terminal ends of the procollagen are important in the alignment and binding of the trimer. The amino and carboxy terminal propeptides are cleaved extracellularly by specific proteinases before the α chains are assembled into fibers.

The portion of the procollagen polypeptide removed from the carboxy terminal, referred to as the carboxyterminal propeptide of Type I collagen, has been found in blood, where its concentration changes during growth and in metabolic bone disorders. Radioimmunoassays for the carboxyterminal propeptide have been reported, (e.g., Taubman et al., Science 186:1115-1117 (1974); Taubman et al., Proc. Soc. Exp. Biol. Med. 152:284-287 (1976); and Parfitt et al. J. Bone Min. Res. 2:427-436 (1987)) including the use of digests of carboxy terminal procollagen obtained from cultured human skin fibroblasts and then purified by digestion, lectin affinity chromatography, gel filtration and ion exchange HPLC (e.g., Meikko et al., Clin. Chem. 36:1328-1332 (1990). These tests, however, are difficult and expensive to prepare and have not found widespread use.

Little work has been reported with the amino terminal propeptide of Type I collagen. In 1987 it was reported that, in a group of patients with primary biliary cirrhosis, both Type I and Type III procollagen aminopropeptides were measured. This was performed by ELISA using purified antigen from calf skin. This study found that the amount of Type I amino propeptide was unchanged,

regardless of the degree of histologic fibrosis on liver biopsy, whereas Type III procollagen varied significantly with disease state (Davis et al., Am. J. Path., 128:265 (1987)).

Accordingly, there remains a need for a sensitive and specific measure of bone formation which is practical to produce and convenient to use. The digests of native procollagen, for example, are not conveniently prepared, do not allow for the production of antibodies to well defined epitopes of interest, and are expensive to develop. The present invention circumvents many of these problems while meeting the requirements for sensitivity and specificity, and fulfills other related needs.

Summary of the Invention

Compositions and methods are provided which are useful in determining the levels of the amino propeptide of human Type I collagen. The levels of the amino propeptide are used as an maker of collagen synthesis in an individual, and thus serve as a sensitive and specific indicator of bone formation. Assays for the amino propeptide find a variety of uses, including use in diagnosing metabolic bone disorders such as osteoporosis or postmenopausal rapid bone losers, monitoring the efficacy of therapeutic regimens designed to treat such disorders, determining the extent of imbalances between bone formation and resorption, etc.

The assays of the invention employ peptides of the amino terminal of procollagen α1 Type I and antibodies specific to said peptides. More particularly, peptides of the invention comprise from six to fifty amino acids and have at least one epitope which immunologically competes with the native amino-terminal propeptide of procollagen α1 Type I. In a preferred embodiment, the epitope(s) is contained in the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp (PEP_{23-34} [Seq. ID No. 1]). The peptides will typically further comprise at least one Cys and/or Tyr residue at the N- or C- terminus to facilitate conjugation, for labeling, and

the like. Other peptides from the amino terminus of the procollagen molecule are also provided. Antibodies, with polyclonal or monoclonal, are provided which bind specifically to a peptide of the invention and thus allow a variety of immunoassay formats. Particularly preferred are radioimmunoassays and enzyme-linked immunosorbent assays to conveniently determine levels of amino terminal procollagen Type I in a sample, such as serum or plasma, obtained from an individual of interest.

Brief Description of the Drawings

Fig. 1 shows antiserum from a rabbit injected with the peptide Seq. ID No. 2 which was diluted and incubated with varying amounts of either the synthetic peptide itself or amino procollagen, alpha 1 (I) which was purified from human skin cells in culture. This shows that both the synthetic peptide and the native amino procollagen displace in parallel and of a similar magnitude.

Fig. 2 represents values obtained from a group of normal subjects and a group of patients with Paget's disease of bone, with a large difference in mean values between the two groups. A break appears in the scale for the y-axis.

Description of the Specific Embodiments

The present invention provides peptides derived from the procollagen alpha (α)1 chain of Type I collagen for use in compositions and methods for the diagnosis, screening and monitoring of bone formation in an individual. The peptides and/or antibodies thereto are useful in assays to determine the level of procollagen α1 (I) activity, and therefore provide a specific and sensitive determination of bone production.

In preferred embodiments the peptides of the invention are derived from amino terminal propeptide region of

the procollagen α1 chain of Type I collagen. The amino terminal propeptide extends from residues 23 to 161 of the proα1 chain of Type I collagen, (residues 1-22 being the signal sequence), where the numbering is according to Tromp et al., Biochem. J. 254:919-922 (1988), which is incorporated herein by reference. Thus, in accordance with the presence invention peptides are provided which contain from six to fifty amino acids of the amino terminal propeptide sequence [Seq. ID No. 11] and which peptides contain at least one epitope that is immunologically competitive with the native amino terminal propeptide. Antibodies produced to the peptides, conveniently synthetically produced peptides, can be used to readily those which contain at least one epitope which is immunologically competitive using well known assay methods. Competition will typically be due to specific binding by the epitopes for binding to the antibody, but in some cases steric hindrance in epitope conformation may also contribute to the competition, as such assays typically measure only the end result regardless of the actual mechanism of competition.

In more preferred embodiments described herein, procollagen peptides (PEP) are derived from the N-terminal regions of amino acid residues 23 to 34 (PEP_{23-34}), residues 28 to 36 (PEP_{28-36}), residues 46 to 53 (PEP_{46-53}), residues 52 to 58 (PEP_{52-58}), residues 76 to 82 (PEP_{76-82}), residues 84 to 91 (PEP_{84-91}; Seq. ID. No. 7), residues 97 to 108 (PEP_{97-108}; Seq. ID No. 8), residues 112 to 121 ($PEP_{112-121}$; Seq. ID No. 9), or residues 130 to 137 ($PEP_{130-137}$; Seq. ID No. 10).

By procollagen α1 Type I "peptide" of the present invention is meant a contiguous chain of at least seven amino acid sequence residues from the procollagen α1 Type I N-terminal propeptide region, sometimes preferably at least eight or nine, sometimes ten to twelve residues, and usually no more than about fifty residues, more usually fewer than about thirty-five, and preferably fewer than twenty-five amino acid residues derived from a selected procollagen α1 Type I propeptide sequence region as set forth herein. The term peptide is used in the present specification to designate a

series of amino acids connected ne to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acids. The peptides can be prepared "synthetically," as described hereinbelow, or by recombinant DNA technology. The peptide will preferably be substantially free of naturally occurring procollagen α1 Type I proteins and fragments thereof. The peptides can be either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the immunoreactivity of the peptide as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the immunoreactivity of a larger peptide. By immunoreactivity is meant the ability of a peptide of the invention to immunologically compete with the amino terminal propeptide of procollagen α1 Type I, and/or which has the ability when used as an immunogen to stimulate the production of antibodies which are capable of specifically binding to the amino terminal propeptide of procollagen α1 Type I.

A preferred immunoreactive procollagen α1 Type I amino terminal peptide of the invention is derived from the N-terminus region, amino acid residues 23 to 34 (PEP_{23-34}). A representative peptide embodiment of this region is the peptide of the following sequence:

PEP_{23-34} [Seq. ID No. 1]

Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp

wherein the peptide can be optionally flanked and/or modified at one or both of the N- and C-termini, as desired, by amino acids from the procollagen α1 Type I amino terminal propeptide sequence, amino acids added to facilitate linking, labeling, other N- and C-terminal modifications, linked to carriers, etc., as further described herein. In a particularly preferred embodiment of PEP_{23-34} the C-terminal further

includes Tyr-Cys residues for convenient labeling, polymerization via disulfide bonds, adsorption, etc., as desired for a particular application. Thus, one PEP_{23-34} peptide so modified has the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp-Tyr-Cys [Seq. ID No. 2] and is described in further detail in the Examples below.

Another procollagen α1 Type I amino-terminal propeptide peptide of the invention comprises at least six contiguous amino acid residues derived from the sequence region of amino acid 28 to 36:

PEP_{28-36} [Seq. ID. No. 3]
Glu-Gly-Gln-Asp-Glu-Asp-Ile-Pro

which optionally includes modifications, including at the N- and/or C- termini, such as additional Tyr and/or Cys residues, as desired.

Yet another procollagen α1 Type I amino-terminal propeptide peptide of the invention comprises at least six contiguous amino acid residues derived from the sequence region of amino acid 46 to 53:

PEP_{46-53} [Seq. ID. No. 4]
Arg-Tyr-His-Asp-Arg-Asp-Val-Trp

which optionally includes modifications, including at the N- and/or C- termini, such as additional Tyr and/or Cys residues, as desired.

Other peptides derived from the amino-terminal propeptide of procollagen α1 Type I include those from the sequence region of amino acid 52 to 58, which has the following sequence:

PEP_{52-58} [Seq. ID. No. 5]
Val-Trp-Lys-Pro-Glu-Pro-Cys

which, again, optionally includes modifications such as at the N- and/or C- termini. One exemplary modification includes a Tyr at the C-terminus, but other modifications are

contemplated within the present invention, as generally set forth herein.

Additional peptides are derived from the amino acid region 76 to 82 of the amino-terminal propeptide procollagen α1 Type I, which region has the sequence:

PEP_{76-82} [Seq. ID. No. 6]
Asp-Glu-Thr-Lys-Asn-Cys-Pro

which optionally includes modifications, including at the N- and/or C- termini, such as additional Tyr and/or Cys residues, and more preferably a Tyr at the C-terminus of peptide PEP_{76-82}, and/or other modifications as desired for the particular application intended.

The invention also includes peptides from the amino acid region 84-91 of the amino-terminal propeptide procollagen α1 Type I, which region has the sequence:

PEP_{84-91} [Seq. ID. No. 7]
Ala-Glu-Val-Pro-Glu-Gly-Glu-Cys

and optionally includes modifications, including at the N- and/or C- termini, such as additional Tyr and/or Cys residues, more preferably a Tyr at the C-terminus of peptide PEP_{84-91}, for example.

Other peptides are derived from the regions of the amino-terminal propeptide of procollagen α1 Type I from residues 97 to 108, 112 to 121, and 130 to 137, which have the following sequences:

PEP_{97-108} [Seq. ID No. 8]
Asp-Gly-Ser-Glu-Ser-Pro-Thr-Asp-Gln-Glu-Thr-Thr

$PEP_{112-121}$ [Seq. ID No. 9]
Gly-Pro-Lys-Gly-Asp-Thr-Gly-Pro-Arg-Gly

$PEP_{130-137}$ [Seq. ID No. 10]
Gly-Arg-Asp-Gly-Ile-Pro-Gly-Gln

which peptides optionally includes modifications, including at the N- and/or C- termini, such as additional Tyr and/or Cys

residues, and/or other modifications as desired for the particular application intended.

As mentioned above, additional amino acids can be added to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier, support or larger peptide, for modifying the physical or chemical properties of the peptide, etc. One or more amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide. In addition, a peptide sequence can differ from the native human amino-terminal propeptide of procollagen α1 Type I sequence by being modified by amino terminal acylation, e.g., acetylation, or thioglycolic acid amidation, carboxy terminal amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

It will be understood that the peptides of the present invention or analogs thereof which have immunoreactivity with the amino-terminal of procollagen α1 Type I may be modified as necessary to provide other desired attributes, e.g., improved immunoreactivity (such as increased immunocompetition with native protein), while increasing or at least not significantly diminishing the immunoreactivity of the unmodified peptide which is derived from the native procollagen sequence. For instance, the peptides may be subject to various changes, such as insertions, deletions, and substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Usually, the portion of the sequence which is intended to substantially mimic an immunoreactive procollagen epitope will not diff r by more than about 20% from the native procollagen sequence, except

where additional amino acids may be added at either terminus for the purpose of modifying the physical or chemical properties of the peptide for, e.g., ease of linking or coupling, and the like.

Having identified different peptides of the invention which are immunoreactive with the procollagen amino-terminal propeptide, in some instances it may be desirable to join two or more peptides in a composition or admixture. The peptides in the composition can be identical or different, and together they should provide equivalent or greater immunoreactivity than the parent peptide(s). For example, using the methods described herein, two or more peptides may define different or overlapping immunoreactive epitopes from different or the same N-terminal procollagen region, which peptides can be combined in a cocktail to provide enhanced immunoreactivity.

The peptides of the invention can be combined via linkage to form polymers. Where the same peptide is linked to itself, thereby forming a homopolymer, a plurality of repeating epitopic units are presented. When the peptides differ, e.g., a cocktail representing different procollagen regions, heteropolymers with repeating units are provided. In addition to covalent linkages, noncovalent linkages capable of forming intermolecular and intrastructural bonds are also contemplated by the present invention.

Linkages for homo- or hetero-polymers or for coupling to carriers can be provided in a variety of ways. For example, cysteine residues can be added at both the amino- and carboxy-termini, where the peptides are covalently bonded via controlled oxidation of the cysteine residues. Also useful are a large number of heterobifunctional agents which generate a disulfide link at one functional group end and a peptide link at the other, including N-succidimidyl-3-(2-pyridyldithio) proprionate (SPDP). This reagent creates a disulfide linkage between itself and a cysteine residue in one protein and an amide linkage through the amino on a lysine or other free amino group in the other.

A variety of such disulfide/amide forming agents are known. See, for example, Immun. Rev. 62:185 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thioether forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2 bromoacetic acid, 2-iodoacetic acid, 4-(N-maleimido-methyl) cyclohexane- 1-carboxylic acid and the like. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxy-2-nitro-4-sulfonic acid, sodium salt. A particularly preferred coupling agent is succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). Of course, it will be understood that linkage should not substantially interfere with the immunoreactivity of either of the linked groups.

In another aspect the peptides of the invention can be combined or coupled with other peptides which present procollagen epitopes, e.g., such as those from the carboxy terminal of the procollagen α1 Type I molecule, some of which are described in European patent publication EP 304,292, incorporated herein by reference.

As mentioned above, amino acid arms may be provided at the C- and/or N-terminus of the peptide or oligopeptide. If present, the arms will usually be at least one amino acid and may be 50 or more amino acids, more often 1 to 10 amino acids, and preferably less than 5 amino acids for ease of synthesis. The arms may serve a variety of purposes, such as spacers, to attach peptides to a carrier, to immobilize peptides to a solid phase, etc. To provide useful functionalities for linking to a carrier, solid phase or to form higher-ordered structures, such as dimers, trimers, or other multimers, amino acids such as tyrosine, cysteine, aspartic acid, or the like, may be introduced at provided at the C- and/or N-terminus of the arm or peptide. To enhance epitope presentation and/or radiolabeling, of particular interest is the presence of from 1 to 10 amino acids at the C- and/or N-terminus, more preferably 1 to 5 amino acids, and

most preferably about 1 to 3. Particularly preferred embodiments of certain peptides described herein are obtained when 3 amino acids are added as an arm, generally at the N-terminus, with the N-terminal residue of the arm preferably Cys. In exemplary embodiments the spacer residues between the peptide and the terminal functional group are Gly. A terminal Cys residue may also be linked through a disulfide linkage to a dithio- or thio-functionalized support or a thioether linkage to an activated olefin support.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984); Tam et al., J. Am. Chem. Soc. 105:6442 (1983); Merrifield, Science 232:341-347 (1986); and Barany and Merrifield, The Peptides, Gross and Meienhofer, eds., Academic Press, New York, pp. 1-284 (1979), each of which is incorporated herein by reference. Short peptide sequences, or libraries of overlapping peptides, usually from about 6 up to about 35 to 50 amino acids, which correspond to the selected regions described herein, can be readily synthesized and then screened in screening assays designed to identify immunoreactive peptides having immunodominant epitopes of the amino-terminal propeptide of Type I procollagen, and the like.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989); Ausubel et al., (ed.) Current Protocols in Molecular Biology,

John Wiley and Sons, Inc., New York (1987), and U.S. Pat. Nos. 4,237,224, 4,273,875, 4,431,739, 4,363,877 and 4,428,941, for example, whose disclosures are incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the determinants of the amino-terminal propeptide of α1 Type I collagen.

As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides and antibodies of the present invention and compositions thereof find use as diagnostic reagents. For example, a peptide as described herein, and/or antibodies to the peptide, may be used to determine the rate of bone formation in an individual. Diagnostic assays for bone formation, often in conjunction with bone resorption assays, can be used to assess net bone balance in an individual. An increase in bone resorption without an adequate compensatory

increase in bone formation may result in osteoporosis or other bone disorder. The amount of free amino-terminal Type I procollagen in a particular individual can be monitored over a period of time and progression or regression of bone formation determined. Samples from an individual can also be compared to relative levels determined from a group of similar patients and the variation therefrom used as a marker of disease progression or regression. Thus, the diagnostic assays provided herein can also be used to assess the responsiveness of an individual to a particular treatment regimen for a bone resorption-related disorder, to modify a treatment protocol, or to establish a prognosis for an affected individual. In addition, the diagnostic assays can be used to predict which individuals will be at substantial risk for developing bone-resorption disorders, such as, e.g., osteoporosis. Also, as skin contributes to the total circulating pool of procollagen peptide, the assays described herein can be used to monitor the condition of burn patients or patients with severe skin lesions.

The antibodies and peptides of the invention also find use in immunocytochemistry, such as to identify osteoblasts or other mesenchymal cells that are producing procollagen, thereby identifying cells which are actively producing collagen to be deposited in the surrounding matrix. Other uses include immunoaffinity purification of procollagen Type I, where immunopurification techniques are generally known in the art and can be adapted for the anti-peptide antibodies described herein to isolate procollagen in substantially pure form, as desired. Patient samples can also be analyzed for the presence of the procollagen Type I amino-terminal using the antibodies prepared against the peptides in Western blot techniques, which are described in U.S. Patent No. 4,452,901, incorporated herein by reference. The antibodies and peptides of the invention can find use in a wide variety of other assays, e.g., screening of genetic libraries, and the like.

As will be recognized by those skilled in the art, numerous types of immunoassays are available for use in the present invention. For instance, direct and indirect binding assays, competitive assays, sandwich assays, and the like, as are generally described in, e.g., U.S. Pat. Nos. 4,642,285; 4,376,110; 4,016,043; 3,879,262; 3,852,157; 3,850,752; 3,839,153; 3,791,932; and Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, N.Y. (1988), each of which is incorporated by reference herein.

The samples to be assayed are derived from an extracellular fluid, cell components or cell products, including, but not limited to, cells and cell culture supernatants, cell extracts, tissue extracts, wound fluids, urine, saliva, blood, plasma, serum, and fractions thereof. As the propeptides of procollagen α1 Type I are believed to be cleaved extracellularly, typically the biological sample will be an extracellular fluid or derived therefrom, although cell surfaces may also be assayed.

The peptide and antibody compositions may be used unlabeled or labeled depending upon their application. By label is intended a molecule which provides, directly or indirectly, a detectable signal. Various labels may be employed, such as radionuclides (e.g., ^{125}I, ^{131}I, ^{3}H, ^{14}C), enzymes, fluorescers, chemiluminescers, enzyme substrates, cofactors or inhibitors, particles (e.g., magnetic particles), combinations of ligands and receptors (e.g., avidin and biotin), dyes, or the like. In addition, the peptides and antibodies thereto may be modified in a variety of ways for binding to a particular surface, such as a microtiter plate, glass or latex bead, tube, filter, chromatographic surface, nitrocellulose paper, cellulose, silica gel, or the like. The particular manner in which peptides and antibodies may be joined to another compound or solid phase surface finds ample illustration in the literature. See, for example, U.S. Patent Nos. 4,371,515; 4,487,715; and patents cited therein. As mentioned above, reagents such as p-maleimidobenzoic acid, p-methyldithiobenzoic acid, maleic acid anhydride, succinic acid

anhydride, glutaraldehyde, hetero-bifunctional cross-linkers, and the like are commonly used for such purposes.

In one assay format, the amount of amino terminal propeptide of α1 Type I collagen in a biological sample is determined in a competition-type assay by measuring the extent that amino terminal Type I propeptide in the sample competes with a peptide of the invention for binding to antibodies specific for the peptide. Although several competitive assay formats are known, in one fluid phase competition assay, such as radioimmunoassay, antibodies and peptides (labeled or capable of being labeled) are allowed to interact in a buffered system under conditions conducive to immune complex formation. A sample suspected of containing the amino terminal propeptide is then added and the system generally allowed to reach equilibrium. The immune complexes resulting from the incubation are subsequently recovered and the amount of label determined, being proportional to the amount of labeled peptides of the invention bound to the antibody. Alternatively, the sample can first be incubated with the antibody and, subsequently or simultaneously, incubated with a labeled peptide of the invention.

In a solid phase competition type immunoassay the primary antibody, which is immunologically reactive with an epitope contained within the sequence of one of the peptides of the invention, such as, e.g., PEP23-34 [Seq. ID No. 1], where the epitope is immunologically competitive with an epitope of amino terminal propeptide of procollagen α1 Type I, is bound, covalently or noncovalently, to a carrier, typically an insoluble solid phase such as a microtiter well. The biological sample to be tested is incubated with the antibody under conditions conducive to immune complex formation, and either simultaneously or subsequently contacted with at least one of the labeled peptides of the invention, also under conditions conducive to immune complex formation. Specifically bound label is then detected, and the presence or quantity of the amino terminal propeptide of procollagen α1 Type I in the sample determined. Typically the amount of

labeled peptide which is bound to the antibodies is proportional (inversely) to the amount of amino terminal propeptide in the sample. Separation steps (e.g., either physicochemical or immunological) and wash steps may be necessary to distinguish specific binding over background.

In an ELISA type immunoassay, again a variety of formats are contemplated. In one method, the synthetic peptide of the invention is bound to a solid phase, e.g. microtiter well, by adsorption, cross-linking, etc. A test sample is incubated with the anti-peptide antibodies of the invention, and then the mixture placed in the peptide-coated well and incubated. Antibody which is not bound by amino terminal propeptide in the sample is bound to the peptide on the solid phase. After a separation step the presence and quantity of bound antibodies can be determined, e.g., using a labeled secondary antibody such as anti-rabbit IgG when the primary antibodies are produced as rabbit antisera, and a convenient color substrate.

Kits can also be supplied for use with the recombinant or synthetic amino terminal procollagen Type I peptides of the invention in determining levels of procollagen turnover in an individual. Thus, the subject peptide compositions may be provided, usually in lyophilized form, in a container, either alone or in conjunction with additional reagents, such as procollagen-specific antibodies, labels, and/or anti-antibodies, and the like. The peptides and antibodies, which may be conjugated to a label, or unconjugated, and are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like. Frequently it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% of the total composition. Where antibodies capable of binding to the procollagen amino terminal and to the peptides of the invention are employed in an assay, they will typically be present in a separate vial.

Monoclonal antibodies for diagnostic uses which bind the amino terminus of human procollagen Type I and peptides of the invention can be produced by a variety of means. The production of murine monoclonal antibodies is well known and may be accomplished by, for example, immunizing the animal with a recombinant or synthetic peptide molecule or a selected portion thereof (e.g., an epitopic domain which competes with an epitope of the amino terminal of human procollagen Type I). Antibody producing cells obtained from the immunized animal are immortalized and screened, or screened first for, e.g., the production of antibody which functions in a competition assay using peptide and amino terminus procollagen molecule, and then immortalized. Antisera (polyclonal antibodies) or monospecific antibodies typically are non-human in origin, such as rabbit, goat, mouse, etc., and can be prepared by immunizing with appropriate peptides, which often will be conjugated to a carrier, e.g., keyhole limpet hemocyanin, for increased immunogenicity. The preparation of antibodies in this manner is well known in the art, such as described in Harlow and Lane, supra.

As mentioned above, various assay protocols may be employed for detecting the presence and/or levels of the amino-terminal propeptide of procollagen Type I in a sample. The peptide may be immobilized either directly or indirectly on a surface, where antibody to the peptide in the sample will become bound to the peptide on the surface. The presence of antibody bound to the peptide can then be detected by employing a xenogeneic antibody specific for the immunoglobulin, normally both IgG and IgM, or a labeled protein specific for immune complexes, e.g., Rf factor or S. aureus protein A or protein G.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I

RIA for Amino Terminal Type I Procollagen

The pro-α chains of type I collagen circulate in large quantities in human sera. Since these chains are a direct result of type I collagen synthesis, their measurement is used a determinant of new bone production.

Antibodies were made to a synthetic peptide of the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp-Tyr-Cys [Seq. ID No. 2]. To produce these antibodies, the peptide was conjugated to KLH using m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as described in Harlow and Lane, supra. The peptide-KLH immunogen was injected into rabbits using standard antibody production protocols. New Zealand White rabbits were injected (Day 1) with 50 μg of conjugated peptide in complete Freund's adjuvant, subcutaneously. Day 21 the rabbits were given 150 μg conjugated peptide in alum by intradermal or intramuscular injection. On Day 49 (4 weeks later), the rabbits received a third injection, same as Day 21. Five weeks later (Day 84) the animal received injections as before. Test bleeds were performed 10 days after the third and subsequent boosts. Assays were performed with the serum from the 4th injection.

Monospecific polyclonal antibodies prepared from the sera of immunized animals were then used to produce a sensitive and specific RIA marker assay for collagen synthesis as determined by circulating Type I pro-α amino terminal procollagen found in serum. The antibodies, in borate buffer, pH 8.4, were used with either a standard (native Type I procollagen amino propeptide or synthetic peptide) or unknown, and tracer molecule (synthetic peptide iodinated with ^{125}I by the chloramine-T method (Hunter and Greenwood, Nature 194:495-496 (1962)) in a total volume of 250 μl and allowed to come to equilibrium overnight at 4°C. Phase separation was accomplished by adding diluted preimmune rabbit serum (typically 100 μl of a dilution to achieve optimal precipitation of the immune sera, usually 1:20 or 1:30),

diluted secondary antibody (goat anti-rabbit IgG, typically 400 µl diluted as with the preimmune sera) and 200 µl of 8% polyethylene glycol. The samples were thoroughly mixed and incubated for three hours at room temperature. Precipitates were collected by centrifugation and bound and total ^{125}I-labeled peptide determined in a gamma counter. This peptide was specific to the peptide fragment in that it did not recognize peptides to the carboxy terminal procollagen or the collagen proteins of other species (sera of rat or dogs were tested).

As shown in Fig. 1, the monospecific antibodies bound to the amino terminal propeptide of type I collagen and the synthetic peptide displaced in parallel to the native protein.

This amino terminal assay in preliminary studies correlated with skeletal alkaline phosphatase ($r= 0.086$, $p< .001$), an indicator of bone formation (Farley et al., Clin. Chem. 27:2002-2007 (1981). It did not correlate with tartrate resistant acid phosphatase ($r= 0.016$, n.s.), a marker for bone resorption. Samples tested were previously collected serum samples from normal children (n=4), adults (n=26), and patients with osteoporosis (n=10), hypoparathyroidism (n=4), or Pagets disease of bone (n=11). These RIAs were performed as above.

EXAMPLE II

ELISA Assay for Amino Terminal of Procollagen

For this ELISA-type assay for circulating immunoterminal of procollagen α1 Type I, the peptide antigen Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp-Tyr-Cys [Seq. ID No. 2] is bound to the bottom of wells of a 96 well plate. This is accomplished by first conjugating the peptide to a different carrier protein or by directly cross-linking the peptide to either an aminated or carboxylated plate (Costar). The test solution (either the titrated peptide, unknown serum

sample, or control) is incubated together with the anti-peptide antibodies described in Example I. After a short incubation period, the mixture is placed in the wells containing the bound peptide, and any free (unbound) antibody is bound to the plate. After washing the presence of bound antibodies is detected using a labeled secondary antibody such as anti-rabbit IgG conjugated to HRP (horseradish peroxidase), then adding a color substrate, such as peroxidase substrate 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) or ABTS. When high concentrations of amino terminal propeptides of α1 Type I collagen are present in the test solution, little antibody will bind to the place, thus the color reaction is low. When little or no antigen is present, the antibodies will bind completely to the antigen on the place, causing a high color state. To quantitate the levels of antigen, the peptide is titrated, making a standard curve to compare against the unknown sample.

A modification of this technique allows that the antibodies are first purified and conjugated to a label (such as HRP or alkaline phosphatase or biotin) directly, thus eliminating problems caused by the extra steps of the secondary reactions.

EXAMPLE III

Measurement of Bone Formation in Patients with Paget's Disease

The RIA described in Example I was used to determine differences in procollagen turnover, and thus hence bone formation, in patients with Paget's disease compared to healthy controls as described in Example I. The results showed a greater than 50-fold difference between serum levels of the amino terminal of procollagen of normals versus serum from the patients with Pagets disease of bone (see Fig. 2). These data support the conclusion that a synthetic peptide which mimics at least one selected epitope in the amino

terminal of procollagen Type I and antibody to the peptide have the ability to measure changes in bone formation rates independent of bone resorption.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Baylink, David J.
Linkhart, Susan

(ii) TITLE OF INVENTION: AMINO PROCOLLAGEN 1(I) PEPTIDES

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Townsend and Townsend
(B) STREET: One Market Plaza, Steuart Street Tower
(C) CITY: San Francisco
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94105-1492

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Parmelee, Steven W.
(B) REGISTRATION NUMBER: 31,990
(C) REFERENCE/DOCKET NUMBER: 14508-3

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (206) 467-9600
(B) TELEFAX: (415) 543-5043

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gln Glu Glu Gly Gln Val Glu Gly Gln Asp Glu Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gln Glu Glu Gly Gln Val Glu Gly Gln Asp Glu Asp Tyr Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Gly Gln Asp Glu Asp Ile Pro
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Tyr His Asp Arg Asp Val Trp
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Val Trp Lys Pro Glu Pro Cys
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asp Glu Thr Lys Asn Cys Pro
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Glu Val Pro Glu Gly Glu Cys
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Gly Ser Glu Ser Pro Thr Asp Gln Glu Thr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Pro Lys Gly Asp Thr Gly Pro Arg Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Arg Asp Gly Ile Pro Gly Gln
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 160 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Phe Ser Phe Val Asp Leu Arg Leu Leu Leu Leu Ala Ala Thr
1 5 10 15

Ala Leu Leu Thr His Gly Gln Glu Glu Gly Gln Val Glu Gly Gln Asp
20 25 30

Glu Asp Ile Pro Pro Ile Thr Cys Val Gln Asn Gly Leu Arg Tyr His
35 40 45

Asp Arg Asp Val Trp Lys Pro Glu Pro Cys Arg Ile Cys Val Cys Asp
50 55 60

Asn Gly Lys Val Leu Cys Asp Asp Val Ile Cys Asp Glu Thr Lys Asn
65 70 75 80

Cys Pro Gly Ala Glu Val Pro Glu Gly Glu Cys Cys Pro Val Cys Pro
85 90 95

Asp Gly Ser Glu Ser Pro Thr Asp Gln Glu Thr Thr Gly Val Glu Gly
100 105 110

Pro Lys Gly Asp Thr Gly Pro Arg Gly Pro Arg Gly Pro Ala Gly Pro
115 120 125

Pro Gly Arg Asp Gly Ile Pro Gly Gln Pro Gly Leu Pro Gly Pro Pro
130 135 140

Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Leu Gly Gly Asn Phe Ala
145 150 155 160

WHAT IS CLAIMED IS:

1. A peptide having from six to fifty amino acids and which has an epitope contained in the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp (PEP_{23-34} [Seq. ID No. 1]), wherein said peptide immunologically competes with amino-terminal propeptide of procollagen α1 Type I.

2. The peptide of claim 1, further comprising at least one Cys or Tyr residue at the N- or C- terminus.

3. The peptide of claim 2, wherein the C-terminal residues comprise Tyr-Cys.

4. The peptide of claim 1, having the sequence Val-Glu-Gly-Gln-Asp-Glu-Asp-Ile-Pro (PEP_{28-36} [Seq. ID No. 3]).

5. The peptide of claim 4, further comprising at least one Cys or Tyr residue at the N- or C- terminus.

6. A method for determining the presence of amino terminal propeptide of procollagen Type I in an individual, comprising:

incubating under conditions conducive to immune complex formation (a) a sample from the individual, (b) an antibody which is immunologically reactive with an epitope contained within the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp (PEP_{23-34} [Seq. ID No. 1]) which epitope is immunologically competitive with an epitope of amino terminal propeptide of procollagen α1 Type I, and (c) a peptide which immunologically binds the antibody and which is labeled to provide a detectable signal, and

detecting said label and therefrom determining the presence of the amino terminal propeptide of procollagen α1 Type I in said individual.

7. The method of claim 6, wherein the peptide is of the sequence

(PEP_{23-34} [Seq. ID No. 1])

X-Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp-X

wherein X is optionally present and comprises when present at least one Cys and or Tyr.

8. The method of claim 6, wherein the sample, antibody and peptide are incubated simultaneouly.

9. The method of claim 6, wherein the incubation of the peptide with the sample and antibody is subsequent to a first incubation of the sample with the antibody.

10. The method of claim 9, wherein the incubation steps are separated by a wash step.

11. The method of claim 6, wherein the antibody is immobilized on a support.

12. The method of claim 1, wherein the support is insoluble.

13. The method of claim 6, wherein the peptide is labeled with an antibody which binds the peptide.

14. The method of claim 6, wherein the sample is human serum, plasma, urine, wound fluid or culture media.

15. The method of claim 6, wherein the label is an enzyme, fluorescer, radionuclide, chemiluminescer or dye.

16. The method of claim 15, wherein the label is ^{125}I or ^{131}I.

17. A method for determining the presence of amino terminal propeptide of procollagen Type I in an individual, comprising:

incubating under conditions conducive to immune complex formation a sample from the individual and an antibody which is immunologically reactive with an epitope contained within the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp (PEP_{23-34} [Seq. ID No. 1]) which epitope is immunologically competitive with an epitope of amino terminal propeptide of procollagen α1 Type I,

incubating, either simultaneous with or subsequent to the incubation of sample and antibody, a peptide bound to a carrier which peptide immunologically binds to the antibody,

separating the immune complexes bound to the carrier from unbound substances,

incubating the separated immune complexes bound to the carrier with an antibody which binds to amino terminal propeptide of procollagen α1 Type I, and

detecting the presence of immune complex formation and therefrom determining the presence of the amino terminal propeptide of procollagen α1 Type I in said individual.

18. A method for monitoring the rate of bone formation in a patient, comprising:

determining over a period of time the relative concentration of the free propeptide of the amino terminal of procollagen Type I in samples from the patient in a method according to claim 6.

19. A test kit for determining the presence of amino terminal propeptide of procollagen Type I in an individual, which comprises:

an antibody which is immunologically reactive with an epitope contained within the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp (PEP_{23-34} [Seq. ID No. 1])

which epitope is immunologically competitive with an epitope of amino terminal prop ptide of procollagen α1 Type I, and a labeled peptide which immunologically binds the antibody.

20. The kit of claim 19, wherein the peptide is of the sequence:

(PEP_{23-34} [Seq. ID No. 1])

X-Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp-Y,

wherein each of X and Y is optionally present and comprises when present at least one Cys and or Tyr.

21. The kit of claim 19, wherein the label is an enzyme, fluorescer, radionuclide, chemiluminescer or dye.

22. The kit of claim 19, wherein the antibody comprises polyclonal antiserum obtained from an animal immunized with a synthetic peptide which contains an epitope within the sequence Gln-Glu-Glu-Gly-Gln-Val-Glu-Gly-Gln-Asp-Glu-Asp (PEP_{23-34} [Seq. ID No. 1]) which is immunologically competitive with an epitope of amino terminal propeptide of procollagen α1 Type I.

Fig. 1

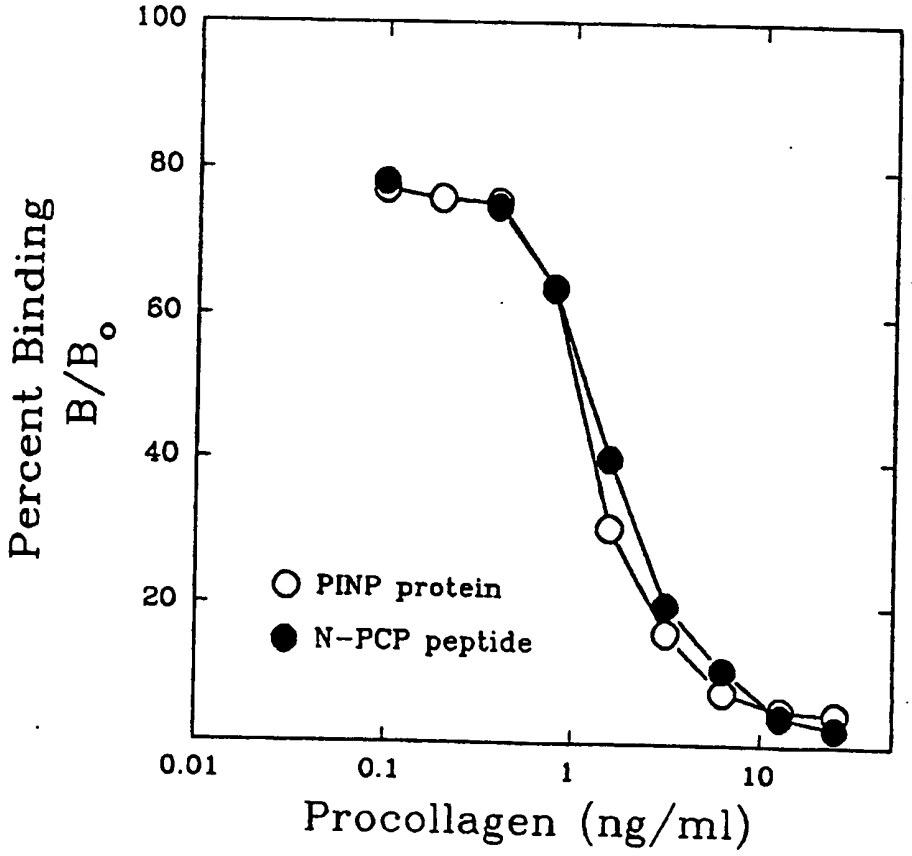
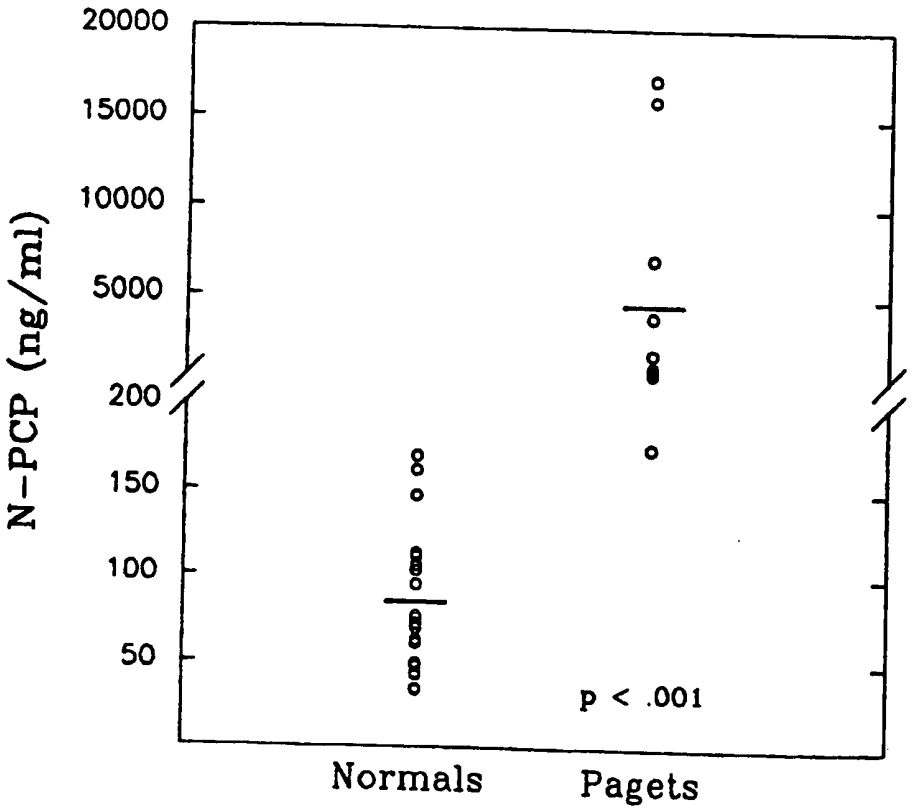

100
80
60
40
20
Percent Binding
B/B$_o$
PINP protein
N-PCP peptide
0.01
0.1
1
10
Procollagen (ng/ml)

Fig. 2


20000
15000
10000
5000
200
150
100
50
N-PCP (ng/ml)
p < .001
Normals
Pagets

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00643

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 7/06, 7/08, 7/10, 15/28; G01N 33/53
US CL :530/300, 356, 387; 435/7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 356, 387; 435/7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, WPI
search terms: aminopeptide, collagen type I, Paget's disease

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	European Journal of Biochemistry, Volume 134, issued 1983, H. G. Foellmer et al., "A Monoclonal Antibody Specific for the Amino Terminal Cleavage Site of Procollagen Type I", pages 183-188, see entire document.	1-22
Y	Nature, Volume 310, issued 26 July 1984, M.-L. Chu et al., "Human proα1(I) collagen gene structure reveals evolutionary conservation of a pattern of introns and exons", pages 337-340, see Figure 4.	1-5
Y	Annual Review of Biochemistry, Volume 47, issued 1978, J. H. Fessler et al., "BIOSYNTHESIS OF PROCOLLAGEN", pages 129-162, see Figure 1 and page 138, lines 12-16.	1-5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 1993	20 APR 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JAMES KETTER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00643

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	American Journal of Pathology, Volume 128, No. 2, issued August 1987, B. H. Davis et al., "An Immunohistochemical and Serum ELISA Study of Type I and III Procollagen Aminopropeptides in Primary Biliary Cirrhosis", pages 265-275, see entire document.	6-22
Y	Gastroenterology, Volume 88, issued May 1985, B. H. Davis et al., "Development of a serum assay of procollagen type I aminopropeptide", page 1655, abstract.	6-22
Y	Journal of Clinical Endocrinology and Metabolism, Volume 58, No. 1, issued 1984, L. S. Simon et al., "Serum Levels of Type I and III Procollagen Fragments in Paget's Disease of Bone", pages 110-120, see entire document.	6-22